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Summary, general discussion and perspectives

This thesis describes the successful biological production and secretion of thioether-stabilized non-lantibiotic peptides. The lantibiotic modification- and transport enzymes NisBTC and LtnM2T involved in the synthesis of the lantibiotics nisin and lacticin 3147, respectively, were exploited for the introduction of thioether bridges in nonlantibiotic peptides. Exploiting the nisin modification enzymes NisB and NisC, we were able to demonstrate for the first time the posttranslational introduction of a thioether bridge in a therapeutic peptide, an analog of angiotensin-(1-7). This therapeutic peptide variant has a significantly improved stability and the effectivity of its interaction with the angiotensin-(1-7) receptor is even enhanced (Chapter 7). Angiotensin-(1-7) plays an important role in counterbalancing many actions of angiotensin II, which is a regulator of fluid and sodium balance, haemodynamics, cellular growth and cardiovascular remodelling (50). Hence, stabilized angiotensin-(1-7) can be a potential therapeutic agent in the control of a range of diseases. Also other methods are used for stabilization of therapeutic peptides and thereby prolonging the half-life of these peptides in the blood. Examples are coupling of peptide drugs to polyethyleneglycol, by modifications such as glycosylation, N-terminal acetylation or C-terminal amidation or by making peptide analogs comprising nonproteinogenic amino acids. While there are hundreds of medically highly important therapeutic peptides, the pharmaceutical market of already a single therapeutic peptide can have a size of over a billion dollar. Consequently, stabilization of already FDA-approved therapeutic peptide hormones and development of new effective stabilized peptides has a tremendous potential. Additionally, via development of a genetically designed peptide library a huge number of biologically produced peptide variants can easily be screened. Utilization of lantibiotic enzymes *in vivo* for introduction of thioether bridges in peptides is therefore a promising technology.

Mechanistic aspects of the enzymes involved in biosynthesis of lantibiotics.

The nisin biosynthesis machinery NisBTC proved to be highly versatile and can be used for the introduction of thioethers in a broad spectrum of nonlantibiotic peptides (Chapter 3, 157, 158). A number of designed nonlantibiotic peptides were

efficiently dehydrated by NisB but failed to be cyclized by NisC, indicating that the substrate specificity of NisB might be more relaxed than that of NisC. Threonines were more easily dehydrated than serines. Furthermore successful dehydration of threonines/serines seems to be influenced by the flanking residues. Hydrophobic flanking residues on one or both sides may favour dehydration, whereas the simultaneous presence of hydrophilic flanking residues on both sides seems to disfavour dehydration (158). Dehydroalanines are highly reactive and can spontaneously form a lanthionine when reacting with a cysteine. On the other hand, methyllanthionines are not formed spontaneously. A methyllanthionine in a microbially produced peptide by utilization of NisBTC is therefore definitely formed by its dehydration by NisB and the subsequent cyclization by NisC. When NisC fails to cyclize a peptide, thioether formation between the dehydrobutyrine and the cysteine can be induced by incubation of the dehydrated peptide at high pH e.g. pH 10. However, non-enzymatic ring closure can result in a mixture of stereoisomers (19).

Thioether linkages can also be introduced by base-assisted sulphur extraction from disulfide bridged peptides (51). Under mild alkaline conditions such chemical introduction of a thioether bridge can result in more than one stereo-isomer (LD, DL, LL and DD). Different isomers can have different retention times when analyzed by HPLC. When biologically produced thioether-stabilized peptides were purified by HPLC, in all studied cases only one peak could be detected. However, when thioether rings were introduced chemically, often more peaks were seen. This suggests that by biological introduction of a thioether bridge, only one isomer, probably the DL variant, is formed. Proof of the presence of only a DL isomer, the only isomer found for the few lantibiotics studied in this respect, can be obtained by Edman degradation of peptides that are desulfurized (52). For lantibiotic-enzyme-cyclized non-lantibiotic peptides, identifying the formed isomer is still one of the future experiments. Not only small thioether-bridged peptides can be synthesized by utilization of NisBTC, also a more complicated substrate peptide with the sequence ITPGCKATVECKITGPCKATVECK can be successfully modified to a peptide with four thioether linkages. Also introduction of intertwined thioether rings is possible thanks to the stereo- and regiospecificity of NisC (160). Moreover, a substrate peptide with the length of 28 amino acids and with the sequence DSRWARVALIDSQKAAVDKAITDIAEKL was produced, with selective dehydration of the threonine at position 22 (157). Especially for longer peptides and peptides with more thioether rings, biological production may have an advantage in reducing the costs and time of synthesis.

Also the lacticin 3147 enzymes LtnT and LtnM2 were successfully used *in vivo* for the introduction of thioether bridges in nonnatural peptide substrates. However, this system is more complex to examine. Secretion of the studied peptides appears to be the bottleneck. Before translocation of the nonnatural substrate by LtnT, the peptide

first has to be processed. The LtnA2 leader is intracellularly cleaved off by the same LtnT enzyme. It is not clear whether the processing or the translocation itself is the restriction in this process (Chapter 4). When introduction of thioether bridges in peptides is performed with lantibiotic enzymes *in vitro*, this processing/ translocation drawback can be avoided.

In 2004 a paper in Science was published that describes the reconstitution of lacticin M *in vitro* for the synthesis of lacticin 481 (226). The enzyme LctM and the substrate LctA were both produced in E. coli. When the His-tagged substrate peptide LctA and the modifying enzyme LctM are incubated *in vitro* in the presence of adenosine triphosphate (ATP) and Mg^{2+}, dehydration and cyclization of the substrate occurs effectively. Though LctM doesn't display an evident ATP-binding domain, ATP is necessary for functionality of LctM (22). Also *in vitro* reconstitution of the two-component haloduracin has been successful. After incubation of the prepeptides HalA1 and HalA2 with the modifying enzymes HalM1 and HalM2, respectively, a bioactive haloduracin is formed (121). Concerning the modifying enzymes NisB and NisC, only NisC has successfully been reconstituted *in vitro*. Incubation of dehydrated prenisin with NisC *in vitro*, results in bioactive nisin after removal of the nisin leader (106).

With the powerful *in vitro* thioether modification system, the substrate specificity and mechanistic aspects of the LctM 481 enzyme were further explored. Just like NisB, LctM has a high substrate promiscuity. LctM can dehydrate a range of nonlantibiotic peptides when attached to the leader peptide (23, 103, 147). While NisB disfavors hydrophilic amino acid residues, especially negatively charged ones, surrounding its dehydratable residues, this is not the case for LctM (23). Furthermore, semi-synthetic LctA derivates with nonproteinogenic amino acids like β-amino acids, D-amino acids and N-alkyl amino acids, derived by expressed protein ligation, are successfully modified by LctM (104, 105, 234). In the case of *in vivo* utilization of lantibiotic enzymes this incorporation of nonproteinogenic amino acids before posttranslational (enzymatic) modification will be very complicated if not impossible. For efficient modification of the structural peptides the precence of the leader peptide is essential. However, it has been demonstrated that presenting the leader in trans, not attached to the substrate, still leads to modification of the structural peptide (102, 147). Moreover, other interesting mechanistic aspects of LctM were revealed. LctM phosphorylates the serines and threonines of its substrate prior to the dehydration of these residues (22, 231). An LctM T405A mutant was not affected in phosphorylation of serines and threonines of its substrate but was hampered in the phosphate-elimination step and therefore lost the ability of dehydration of its substrate. This mutant shows to be a highly efficient kinase for a broad range of peptide substrates with serines when preceded by the lacticin leader (232). Recently, LctM-mediated dehydration was demonstrated to occur via a distributive mechanism (96).

Furthermore a tendency to an apparently directional behavior of the LctM enzyme was revealed (96). LctM has a high, though not strict, propensity for an apparent N-to-C directionality. When the leader peptide is present in trans, dehydrations were nondirectional. Also intermediates were found which were not completely dehydrated but already contained rings within their N-terminal region. This latter finding suggests that the dehydration - and the cyclization activities of LctM can alternate. This alternating feature was also observed for NisB- and NisC activity, outlined in this thesis in Chapter 4. The latter data were supported by data obtained by Lubelski et al., who also suggested that NisB and NisC are acting in an N- to C- direction (114).

Chapters 4 and 5 of this thesis demonstrate that the nisin synthetase complex and the lacticin 3147 synthetase complex can be dissected and that enzymes still can be functional without the presence of other lantibiotic enzymes. For the nisin biosynthetic enzymes it was demonstrated that NisT can transport peptides when preceded by the nisin leader in the absence of all other nisin enzymes. The same is true for the dehydratase activity of the nisin dehydratase NisB. In the absence of other nisin enzymes, NisB-dehydrated peptides were exported via the Sec pathway when the nisin leader was preceded by a Sec signal sequence. When the same peptide substrate was preceded by a Tat signal sequence and exported via the Sec pathway, an identical level of dehydration of the peptide was obtained compared to the case of dehydration and export via NisBT. Moreover prenisin without preceding Sec or Tat signal was intracellularly fully modified by NisB and NisC in the absence of NisT, which precludes the necessity of either NisT or Sec action.

In view of the above it is difficult to comprehend the proposed enzyme-complex-dependent NisT-driven-modification working model for nisin biosynthesis by Lubelski et al. 2009 (114). In this model it was speculated that NisT, that uses ATP for the transport, is also involved in pulling the substrate through the active sites of the modification enzymes NisB and NisC.

Production and transport of posttranslationally modified peptides.

The ABC transporter NisT is capable of translocating a broad range of substrates. The substrates that have been examined up to now, varied in size from 6 amino acids up to 47 amino acids, were linear, dehydrated and/or had various (intertwined) thioether rings. Also the leader peptide itself, without any propeptide was translocated (158). Although the NisT transporter is very successful in translocation of nonnatural substrate peptides, not all tested peptides are transported equally well via NisT. Whether these differences in transport efficiency are due to size, polarity, hydrophobicity or conformation of the peptides is not established yet. Furthermore, it was noticed that fusion of peptides of interest behind the first two rings of nisin improved the production level significantly (161). This improvement can be caused by

the autoinducing effects of rings A and B of nisin (91) or may be based on the positive effect of these rings on translocation via NisT.

Another alternative route for transport of modified peptides in *L. lactis* is the Sec pathway, which is successfully demonstrated in Chapters 5 and 6 of this thesis. For transport of peptides or proteins via the well studied Sec pathway substrates have to be preceded by a Sec signal peptide. It was demonstrated that an N-terminal fusion to the nisin leader of a signal peptide up to 44 amino acids long didn't prevent dehydration by NisB and cyclization by NisC. This feature has a high potential. For instance, N-terminal extensions to the leader peptide can be a promising tool in the development of combining the thioether modification technology with phage display. With these united techniques huge libraries of thioether-constrained peptides can be made and screened for dedicated purposes. Furthermore, the Twin arginine signal sequence of YwbN still targets the peptide to the Sec pathway in *L. lactis*. This targeting signal sequence had a positive effect on the dehydration efficiency possibly thanks to a reduced translocation efficiency of the peptide. By altering the signal sequence a more optimal system may be created with improved modification and transport efficiency. Although the Sec pathway can successfully transport linear dehydrated peptides and an azurin peptide fragment with a small methyllanthionine, the route was not fuctional for producing prenisin. It was estimated that the size of the SecY pore in *L. lactis* is too small for translocation of prenisin. In E. coli it has been demonstrated that the translocon SecYEG could also transport the polypeptide proOmpA with disulfides or labeled with a bulky fluorescent probe (33, 206). Possibly in the future improved transport via the Sec pathway in *L. lactis* can be obtained by making SecY mutants with a a more relaxed fitting that are able to transport also more bulky peptides.

Studies on lantibiotic biosynthesis systems for nisin, subtilin and nukacin ISK1 revealed that the modification enzymes and transporters are arranged in multimeric membrane-associated enzyme complexes (75, 133, 189). A nisin synthetase complex composed of two NisT molecules, two NisC molecules and one NisB molecule in the WT strain has been suggested. Whether this stoichiometry of enzymes is also present in the two plasmid production strain that we used for the production of modified peptides has not yet been determined. The optimal stoichiometry of substrate, modification enzymes and transporter probably results from a fine-tuned balance in expression. Raising the intracellular concentration of NisB and thereby changing the ratio substrate: NisB, led to an improved dehydration level of the secreted peptides (72, Chapter 5). Additionally, in a study of van den Berg van Saparoea et al. a channeling of prenisin of NisB to NisT was suggested to obtain efficient transport of prenisin. In the presence of the enzymes NisB and NisC a higher production level of prenisin was found when compared with the production level of the dehydrated prepeptide NisA in the absence of NisC. Moreover, in the absence of NisB and NisC

the production level of unmodified precursor NisA was even lower (211). These data underline the presence of a multimeric enzyme complex and the importance thereof for optimal production of prenisin. However, in the absence of the cyclase NisC thioether rings are absent. The presence of thioether rings might impose a structure which is transported more efficiently by NisT. The latter possibility is revealed by data outlined in Chapter 4 of this thesis. Furthermore, rings A and B are involved in induction of the Pnis promoter. Even a low extent of removal of the leader by peptidases can have an impact on production of prenisin by autoinduction.

The technology described in this thesis is unique, especially with respect to the regio-, stereo- and chemospecific synthesis. After the production of the successful thioether angiotensin-(1-7), challenging applied perspectives are now the selection of new relevant candidate substrate peptides for thioether stabilization. Improvement of production levels of peptides of interest may be obtained by several adjustments of the biological production system. Examples for reaching the latter goal are: using another production host / host engineering, raising the amount of the intracellular substrate and modification enzymes by raising the copy numbers of the encoding genes, adjusting the nisin leader or the NisT transporter for better transport or improving growth conditions of the producing culture.

From the more fundamental point of view, the lantibiotic enzymes constitute a fascinatingly rich and diverse field of research. Important new mechanistic insights may follow from the crystallization of lantibiotic enzymes other than NisC. *In vitro* reconstitution of NisB activity, which has not been attained despite many efforts in several laboratories, will hopefully be realized one day and facilitate further mechanistic studies.